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| (51) International Patent Classification ⁶: C12Q 1/68, C07H 21/00, 21/04 | A1 | (11) International Publication Number: WO 99/13109 (43) International Publication Date: 18 March 1999 (18.03.99) |
| (21) International Application Number: PCT/US98/18808 (22) International Filing Date: 10 September 1998 (10.09.98) (30) Priority Data: 60/058,642 11 September 1997 (11.09.97) US (71) Applicant (for all designated States except US): SEQ. LTD. [US/US]; 17 Princess Road, Lawrenceville, NJ 08648 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MACKLIN, John, J. [US/US]; 104 North Monroe Avenue, Wenonah, NJ 08090 (US). TRAUTMAN, Jay, K. [US/US]; 28 East Welling Avenue, Pennington, NJ 08534 (US). HARRIS, Timothy, D. [US/US]; 1387 Church Road, Toms River, NJ 08755 (US). (74) Agents: MORRIS, Francis, E. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US). | | (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> |
| (54) Title: METHOD FOR DNA SEQUENCING ANALYSIS | | |
| (57) Abstract Cleaved nucleotides are affixed to a substrate so that each nucleotide is located at a different position on the substrate in the same order in which it was cleaved. The affixed nucleotides are then contacted with reagents that selectively convert one or more of the different types of nucleotides in place to products that have enhanced fluorescence. Where more than one type of nucleotide is so converted, the conversion product for each type of nucleotide advantageously has a fluorescence characteristic e.g., wavelength of maximum absorption or emission, or fluorescence lifetime) that is different from the fluorescence characteristic of the other conversion products. The conversion products are illuminated with electromagnetic radiation at a wavelength that is suitable for causing the conversion product to fluoresce. The fluorescence of each conversion product is detected as a function of the position of the conversion product on the substrate. Where the fluorescence of each conversion product is distinctive, detection of all the conversion products can be done simultaneously. Where the fluorescence of two or more conversion products is not distinctive, each such product should be detected at a different time. The result of the foregoing process is a map that indicates the position on the substrate of the different conversion products that were caused to fluoresce. Since the position of the conversion products corresponds to the position of the nucleotides that were affixed to the substrate and since the nucleotides were deposited on the substrate in the sequence in which they were cleaved, the map identifies the position in the oligonucleotide of those nucleotides that were converted to conversion products whose fluorescence was detected. | | |

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METHOD FOR DNA SEQUENCING ANALYSIS

Summary of the Invention

Methods are known for using an exonuclease to cleave
5 nucleotides one-at-a-time from an oligonucleotide such as DNA
to which the exonuclease is attached. The present invention
is a method for identifying one or more of the different
types of nucleotides in the resulting sequence of cleaved
nucleotides.

10 In accordance with a preferred embodiment of the
invention, the cleaved nucleotides are affixed to a substrate
so that each nucleotide is located at a different position on
the substrate in the same order in which it was cleaved. The
affixed nucleotides are then contacted with reagents and may
15 be illuminated with electromagnetic radiation so as to
selectively convert one or more of the different types of
nucleotides in place to products that have enhanced
fluorescence. Where more than one type of nucleotide is so
converted, the conversion product for each type of nucleotide
20 advantageously has a fluorescence characteristic (e.g.,
wavelength of maximum absorption or emission, or fluorescence
lifetime) that is different from the fluorescence
characteristic of the other conversion products.

The conversion products are illuminated with
25 electromagnetic radiation at a wavelength that is suitable
for causing the conversion product to fluoresce. The
fluorescence of each conversion product is detected as a
function of the position of the conversion product on the
substrate. Where the fluorescence of each conversion product
30 is distinctive, detection of all the conversion products can
be done simultaneously. Where the fluorescence of two or
more conversion products is not distinctive, each such
product is formed and detected at a different time.

The result of the foregoing process is a map that
35 indicates the position on the substrate of the different
conversion products that were caused to fluoresce. Since the
position of the conversion products corresponds to the

position of the nucleotides that were affixed to the substrate and since the nucleotides were deposited on the substrate in the sequence in which they were cleaved, the map identifies the position in the oligonucleotide of those
5 nucleotides that were converted to conversion products whose fluorescence was detected.

Preferably all four nucleotides are converted to conversion products having distinctive fluorescence. However, the invention may also be practiced where only one,
10 two or three nucleotides are converted to conversion products having distinctive fluorescence. While information relating to the location of only one, two or three nucleotides in an oligonucleotide sequence may not be complete, it is still useful. Moreover, in the case of DNA sequencing, where
15 information is available about at least a non-complimentary pair of bases such as adenine and guanine or about cytosine and thymine, information as to the position of the other two bases can be obtained by sequencing the complementary strand.

20 Brief Description of the Drawings

These and other objects, features and advantages of the invention will be more readily apparent from the following detailed description of the invention in which:

Fig. 1 depicts a system useful in cleaving nucleotides
25 one-at-a-time from an oligonucleotide;

Fig. 2 (a) is an enlarged side view of a portion of the system of Fig. 1 depicting the cleavage of nucleotides one-at-a-time from an oligonucleotide;

Fig. 2 (b) is a top view of the same portion of the
30 system depicted by Fig. 2 (a);

Fig. 2 (c) is a top view of a substrate to which the cleaved nucleotides have been affixed;

Fig. 3 depicts a system for detecting the fluorescence from conversion products formed in accordance with the
35 invention;

Figs. 4A-4E depict illustrative maps of the position of the nucleotides on the substrate;

Fig. 5 depicts an alternative system for forming a sequence of nucleotides on a substrate;

Fig. 6 is a schematic representation of several chemical compounds; and

- 5 Fig. 7(a), 7(b) and 7(c) show fluorescent spectra for two modified nucleotides illuminated at two different wavelengths.

Detailed Description of the Drawings

- 10 Fig. 1 depicts an optical trap useful in practicing a preferred embodiment of the invention. Fig. 1 is reproduced from U.S. Patent 5,079,169 which is incorporated herein by reference and the elements of Fig. 1 bear the same reference numerals.

- 15 Fig. 1 depicts a conventional optical trap 10 of the type which is used in practicing the invention. The trap comprises a modified fluorescence microscope 12 including a chamber 14 containing a liquid cell where particle manipulation takes place. The chamber is mounted on a
20 conventional microscope stage 16 which can be moved in two orthogonal directions in the plane perpendicular to the axis of the microscope, as well as along the optical axis.

The optical trap is formed by a laser beam from a laser 22 which is focused on chamber 14 by a highly convergent
25 objective lens 48. Illustratively, the laser is an argon ion laser, a diode laser or a NdYAG laser. Lens 48 typically has a numerical aperture greater than 0.8 and preferably about 1.2 or greater. Advantageously, lens 48 is a liquid immersion type and an oil drop between lens 48 and the cover
30 of chamber 14 approximately matches the refractive indices of the lens and the cover so as to minimize light losses at the surfaces.

The position of the optical trap in chamber 14 may be moved by moving platform 24 in the X or Y directions.
35 Alternatively, stage 16 may be moved with respect to the optical trap.

The apparatus of Fig. 1 further comprises an image intensified video camera 60 or other electro-optical imaging device, a fluorescence light source 62 such as an argon laser or a mercury lamp and a visible light source 66.

5 Further details concerning this optical trap are set forth in the '169 patent. A similar such trap is illustrated in Fig. 1 of U.S. Patent 4,893,886 which is likewise incorporated herein by reference.

As described in PCT Patent Application No. PCT/96/08633,
10 filed June 4, 1996, which is likewise incorporated herein by reference, an optical trap may be used to draw an oligonucleotide through a thin film on a substrate. In particular, an oligonucleotide such as a strand of DNA may be attached at one end to a small bead using known methods and
15 the bead may be secured in the trap. The bead and oligonucleotide may then be drawn through the thin film by moving the substrate relative to the trap, by moving the trap, or by moving both. This is depicted in Fig. 2 (a) and 2 (b) which show a thin film 80 on a substrate 82, an optical
20 trap 84 in which is secured a bead 86 and an oligonucleotide 88 attached to the bead.

By attaching a processive exonuclease 90 to the free end of the oligonucleotide and providing conditions in the thin film such that the exonuclease is activated, individual
25 nucleotides 92 will be cleaved from the oligonucleotide in the same order in which they are found in the oligonucleotide. Absolute position of the nucleotides is referenced to unique position markers 94 attached to the surface. By ensuring that the bead and oligonucleotide move
30 continuously through the thin film in a path that does not cross itself, each of the cleaved nucleotides is deposited in the film at a different position but in the same order in which it is found in the oligonucleotide. Advantageously, a microchannel is defined in the surface of the substrate and
35 the oligonucleotide is drawn through a thin film in this channel. The channel minimizes lateral diffusion of the

nucleotides and therefore aids in defining their position on the substrate.

In accordance with the invention, each of the cleaved nucleotides is affixed to the substrate in the order in which
5 it was deposited in the film. Advantageously, this is effected by electrostatic attraction between the substrate which advantageously is made of alumina and the negative phosphate group of the nucleotides. It may be further promoted by an applied electric field or other means
10 discussed below.

Fig. 2 (c) depicts substrate 82 to which a sequence of nucleotides has been affixed.

After the bases have been affixed to the substrate, one or more of the bases and preferably all four are converted to
15 conversion products having a fluorescence that is substantially enhanced over that of the native nucleotide. As described in greater detail below, this conversion is preferably effected by contacting the substrate with solutions containing suitable reagents and, when the
20 conversion product is formed from a photochemical reaction, illuminating the substrate with uv radiation. This can be done using a separate solution for each of the four nucleotides followed by a washing step or it may be possible to combine in one solution suitable reagents for converting
25 more than one of the nucleotides to the desired conversion products.

The conversion products are then detected using the apparatus of Fig. 3 which is essentially a microscope with a CCD readout. As shown in Fig. 3, this apparatus comprises a
30 substrate 82 to which the conversion products are affixed, a quartz coverslip 115, a high numerical aperture objective lens 120, a dichroic mirror 125, first and second CCD cameras 130, 135 and a CPU 140 for controlling the microscope and processing the data received by the CCD cameras.
35 Advantageously, the substrate is covered with a detection solution 112 which typically is an aqueous buffer solution free of dissolved oxygen and containing a triplet quencher.

The substrate is mounted on a microscope stage which is movable in at least one dimension and, in particular, is movable along the same pathway that the oligonucleotide was drawn when the nucleotide were cleaved from the oligonucleotide and affixed to the substrate.

Advantageously, the movement of the microscope stage is controlled by CPU 140.

The substrate is illuminated from below by a source of electromagnetic radiation, preferably a laser (not shown), having a wavelength suitable for stimulating fluorescence in the conversion products affixed to the substrate. A suitable laser is a mode-locked Ti:sapphire laser, frequency-tripled into the ultraviolet, that produces tunable wavelengths from about 260 nanometers (nm) to 295 nm with 100 to 200 milliWatts (mW) of power. Alternative sources may be an Ar-ion laser which is capable of producing 3 mW at 275 nm and 10 mW at 300 nm and a Hg lamp which produces about 5 mW at 240-260 nm.

Electromagnetic radiation is evanescently coupled to the conversion products so that the intensity of the radiation decreases exponentially with distance into the detection solution. As a result, any absorbing molecules in the detection solution are minimally excited, thereby minimizing unwanted background fluorescence. This also allows molecules to interact with the bound conversion products and not be depleted by the ultraviolet light.

The microscope is set to image a portion of the substrate at a time, about a 250-micron field of view for a 100x objective. A slightly smaller fraction of the field is illuminated by the uv light. The resulting spatially isolated fluorescence from the photoproducts is imaged onto a CCD camera, with suitable optical filters placed in the detection path to preferentially pass only the wavelengths of interest. Typically the uv intensity is about 50 W/cm². The CCD camera and the uv light is shuttered on for about one second then shuttered off. This produces about 50 photons

per molecule detected as spatially resolved spots on the CCD image, or frame.

The substrate is then moved in the direction that the original DNA strand was dragged, by an amount slightly
5 smaller than the field of view, so that the uv excitation light slightly overlaps the previous field and the present field of view; and the lumination process is repeated.

The apparatus of Fig. 3 may be used in a variety of ways to detect the conversion products on substrate 82. Ideally,
10 all four conversion products will have sufficiently different fluorescence emission characteristics that they can be detected simultaneously. As an aid to this type of operation, both CCD cameras may be used for simultaneous
detection with different wavelength filters 132, 137 inserted
15 in their optical paths. This approach has the advantage of avoiding registration problems that otherwise might occur when combining two or more successive images of the conversion products on substrate 82.

However, the invention may also be practiced by
20 monitoring the fluorescence of only one, two or three of the conversion products at one time and then combining the images that are generated. Monitoring the fluorescence of only one conversion product at a time has the advantage that the
illuminating radiation may be tuned to a wavelength that has
25 maximum absorption by only one of the conversion products, thereby enhancing the fluorescence from that conversion product and improving its detectability by the CCD camera.

In the case where each conversion product is separately illuminated, four images are produced which must then be
30 combined to produce a single map depicting the sequence of the nucleotides as affixed to the substrate. For the case of the sequence of nucleotides deposited on substrate 82, four illustrative images that would be detected are shown in Figs. 4A, 4B, 4C and 4D for the four bases A,C,G and T and the
35 combined image is illustrated in Fig. 4E.

Other spectroscopic characteristics of the conversion products can be additionally used for discrimination in the

arrangement shown in Fig. 3. For example, it may be desirable to time-resolve the fluorescence from the nucleotides, since the fluorescent lifetime may be a unique characteristic of the modified nucleotides. As indicated in 5 Fig. 3, a sample containing the conversion products is illuminated with electromagnetic radiation that causes the products to fluoresce, where the illumination is from a pulsed light source, for example a mode-locked laser, where the pulse duration is shorter than the shortest fluorescent 10 decay of the modified nucleotides, while the time between pulses is much longer than the longest fluorescent decay. As above, upon illumination, a fluorescence image of the sample is formed on a time-resolved fluorescence detector from a unique spatial location in the field of view. In software 15 processing, the decay time for each channel (or spatial location) is determined, and a fluorescence-lifetime image is produced. This image can then be used to determine the spatial location and identity of a conversion product, based on the lifetime recorded at each pixel. This method of 20 discrimination may be used in addition to spectral filtering to provide a higher confidence level in the unique identification of a conversion product.

The detection arrangement described above could be modified to incorporate confocal illumination and detection, 25 where the uv illumination beam is focused to a small spot on the sample, and all the fluorescence emission from the sample at that spot, after suitable optical filtering, is sent to a single channel detector, for example, a MCP detector or an avalanche-photodiode. In this approach, the fluorescence 30 decay at each spot on the sample is analyzed by software to determine the presence and identity of the conversion product, based on the optically-filtered fluorescence decay time. The sample is rastered so that all spots on the sample are probed. This detection scheme can be extended to 35 incorporate a slit confocal geometry, which is intermediate between a full-field geometry and a single-point confocal geometry.

Numerous alternatives may be used in the practice of the invention. Rather than cleave the nucleotides from an oligonucleotide that is being drawn through a thin film, other arrangements may be used for separating the
5 nucleotides. For example, the oligonucleotide may be suspended in a flowing stream of solution with an active exonuclease attached to its free end. See, for example, U.S. patent application Serial No. 08/376,761, which is incorporated herein by reference. In this situation, cleaved
10 nucleotides become entrained in the flowing stream and may be deposited in sequence on a substrate by apparatus such as that shown in Fig. 5. Again, this apparatus establishes on the substrate a sequence of nucleotides in the order in which they were cleaved from the oligonucleotide.

15 As shown in Fig. 5, the flowing stream is deposited by a nozzle 300 in a thin continuous liquid film 305 or as discrete droplets on a transparent support 310. The liquid film or droplets are then solidified on the solid support by cooling the support and/or polymerization and/or drying.
20 Finally, the film is transported by movement of support 310 through a detection station 320 where it is irradiated by a radiation source 325; and the resulting fluorescence is detected by detection system 330 and identified by computer 335. The specific structure of detection station 320 may be
25 similar to the apparatus of Fig. 3.

The support can take the form of any surface geometry which can be moved with respect to the output nozzle 300 so as to allow for the deposit of the nucleotide containing liquid stream in such a manner that the position of deposit
30 of each nucleotide is both unique and known (Merrill et al., 1979, J. Histochem. Cytochem. 27:280-283, which is incorporated herein by reference). Uniqueness requires that individual nucleotides are deposited on the surface with sufficient distance between each nucleotide and any other
35 nucleotide so as to be isolated in an optically-resolvable volume element during the subsequent step of nucleotide detection and identification *infra*. The sequential position

of deposit of each nucleotide must be known with sufficient accuracy so as to be able to position the volume element containing each nucleotide with respect to the excitation volume of the detection system *infra*.

- 5 The detection of nucleotides in a continuous film 305 or in discrete droplets on a support 310 is similar to the detection of nucleotides in the apparatus of Fig. 3. As indicated in Fig. 5, film 305 (or droplets) is moved through a beam of radiation from a radiation source 325 and
- 10 fluorescence is detected by a detector system 330. However, in this case the cross-sectional dimension of film 305 (or of the droplets) is almost certain to be much greater than that in the apparatus of Fig. 3. In such case it will be advantageous to scan the laser beam transversely to the
- 15 direction of motion of the film (or droplet) through the detection station. Such scanning motion can readily be implemented by directing the laser beam at a rotating mirror such that the reflected beam sweeps across the path of the moving film.
- 20 One advantage of the approach of Fig. 5 is that it provides additional possibilities for performance of the step of converting the nucleotides to conversion products with enhanced fluorescence. In particular, one or more reagents for effecting the conversion can be introduced into the
- 25 flowing stream upstream of the nozzle. If the activity of the exonuclease is not inhibited by such reagents, the reagents can be introduced into the stream upstream of the exonuclease. Otherwise, they can be introduced downstream of the point where the nucleotides are cleaved from the
- 30 oligonucleotide.

While it is preferred that all of the nucleotides first be converted to conversion products before any of the conversion products are illuminated for detection, the invention may also be practiced by performing one or more

35 conversions, illuminating the substrate to detect one or more of the conversion products and then converting one or more other nucleotides to conversion products.

Binding and Immobilization of Nucleotides to Surfaces

The immobilization of monophosphate nucleotides at a liquid/solid interface following their sequential cleavage from a strand of DNA represents a useful method for their subsequent detection at the single molecule level. It is known that metal oxides such as aluminum oxide (alumina) preferentially binds nucleotides but not nucleosides or bases¹⁻³ where binding results from the electrostatic attraction of the negative phosphate group of a nucleotide with the tri-valent positively charged aluminum ions on the alumina surface. Because the binding occurs through the phosphate group all of the monophosphate nucleotides (dAMP, dCMP, dGMP, and TMP) bind with nearly equal binding affinity. Examples of possible coordination of phosphate on alumina are discussed in Refs. 4, 5. Other surfaces, particularly monolayer films applied by the Langmuir-Blodgett technique, can be used. For example, to bind monophosphate nucleotides, we have used an aluminum-alkanebisphosphonate thin film such as that used to immobilize DNA⁶.

Surface coverage assays using scintillation of ³²P-labeled dAMP, or fluorescence detection of dye-labeled nucleotides (fluorescein-labeled dUTP), or fluorescent-analog nucleotides (2APTP, etheno-dAMP, dGMP-photoproduct) show that the alumina surface adsorbs up to about 10⁴ nucleotides per square micron (about 1% of a monolayer), which is the apparent saturation coverage. At these coverages, dimer formation on the surface is not a significant factor, so that the photophysical properties measured will reflect the behavior of monomer nucleotides, and can be extrapolated to low surface coverage, i.e., single surface-bound nucleotides. By comparison, the nucleotide binding efficiency to a quartz surface with no alumina is down about 10,000-fold.

We have shown that alumina-bound nucleotides, overcoated with water, can remain bound to the surface for hours, with a 1/e off-rate of about 1/(7 hrs) at room temperature. This could be further reduced using a less polar solvent to overcoat the nucleotides. Once the nucleotides are bound to

the surface, changing the solution buffer does not appear to displace the nucleotides from the surface, unless a very acidic solution or concentrated phosphate-buffer solution is used.

5 Photophysical measurements show that the fluorescence properties of surface-bound nucleotides are essentially unchanged from those found in solution. There is no indication of undesirable processes such as charge- or energy-transfer from the nucleotide to the surface. The
10 generic binding of nucleotides through the deoxyriboside-monophosphate moiety and not by linkage through the purine or pyrimidine base ring structure allows the solution-like fluorescence properties. These findings agree with those of Ref. 7 that the photochemistry (and the photophysics) on
15 oxide surfaces are reminiscent of reactions in polar homogeneous solutions (e.g., water).

After completing the DNA-digestion process, the substrate containing the spatially-ordered surface-immobilized nucleotides can be processed to further decrease
20 the off-rate and diffusion of the bound nucleotides. For example, a solution containing 'blocking' molecules that bind to the alumina can be spread over the surface. A blocking molecule could be riboside-monophosphate (a nucleotide without a base) that binds up the remaining available sites
25 on the alumina surface, without displacing the nucleotides. The blocking molecules act to (1) prevent binding of reagent molecules in subsequent processing steps, and (2) further reduce the diffusion of nucleotides along the surface (by analogy to the restricted movement of cars in a filled
30 parking lot). Once the excess alumina sites are tied up with blocking molecules, this solution can be washed off, and replaced with another. The sample is then ready for the process of fluorescence enhancement and detection of the surface bound nucleotides, or the substrate can be stored at
35 low temperature indefinitely, with no displacement of the nucleotides, until needed for the detection process.

Fluorescence Enhancement of Nucleotide Fluorescence

A method to enhance the fluorescence properties of native nucleotides preferably does not involve the nucleotide phosphate group, which is used to bind the nucleotide to a substrate. This can be accomplished by a chemical or photochemical reaction between the nucleotide and a non-fluorescent reagent that results in a fluorescent product. This product is a uv-excited, near-uv emissive fluorophore with high quantum yield. This method does not require aggressive removal of the reagent after the reaction; the non-fluorescent reagent will not obscure the visibility or detectability of the fluorescent product.

Figure 6 shows the molecular structure of the four bases of DNA, along with four fluorescent products following chemical modification of the bases. The spectroscopic properties of the fluorescent products are listed in Table I. For example, a particularly simple chemical synthesis is the reaction of chloroacetal with dAMP to form the highly fluorescent (quantum yield of 56%) 1,N⁶-ethenoadenosine monophosphate, or etheno-dAMP. This reaction in solution proceeds to complete conversion with no side reaction in excess chloroacetal. We have verified that the conversion reagents do not generate appreciable background when applied to a crystalline alumina binding surface.

In addition to the chemical conversion of native nucleotides to fluorescent products, we have also shown it is possible to use a photochemical reaction to accomplish the conversion. See, our application "Method to Make Fluorescent Nucleotide Photoproducts for DNA Sequencing and Analysis," filed simultaneously herewith and incorporated herein by reference. For example, when deoxyguanosine 5'-monophosphate (dGMP) is illuminated with electromagnetic radiation in the presence of certain reagents such as glycerol, a highly fluorescent photoproduct is formed. This photoproduct is spectroscopically very similar to 2-aminopurine, shown in Fig. 6 and listed in Table I. When combined with the chemical modification of dAMP to make the fluorescent etheno-

dAMP, an attractive set of fluorophores can be obtained for application to DNA sequencing by detection of single molecules.

The methods for chemical and photochemical conversion
5 can be ordered to optimize the fluorescence detection process while minimizing the decomposition of the product molecules. For example, the photochemical conversion of dGMP to the dGMP-photoproduct will not produce significant photodecomposition of dAMP. This may be accomplished by
10 choice of the wavelength of the radiation used for the photochemistry. The dGMP nucleotide may be photoconverted to the dGMP-photoproduct using, for example, 280 nm light, which lies outside the absorption band of dAMP and has little deleterious effect on dAMP nucleotides. A map of the spatial
15 location of the dGMP-products can be obtained. Then a chemical modification step is employed to convert dAMP to etheno-dAMP, where chemistry used may produce little modification of the dGMP-photoproduct.

Alternatively, the photochemical and chemical conversion
20 may be first performed, followed by the detection and discrimination of the conversion products in a subsequent step. As an example, Fig. 7(a) shows the emission and excitation spectra for a dGMP photoproduct made by photochemical reaction, and for etheno-dAMP, the chemically-
25 modified analog of dAMP. It can be seen that the two emission spectra are distinguishable, so that using excitation at, say, 300 nm, the dGMP-photoproduct can be distinguished from the dAMP-product by simple optical filters in front of the detector(s). For even greater
30 discrimination, a sample could first be excited at 265 nm, where the etheno-dAMP absorbance is maximum and the dGMP-photoproduct absorbance is minimum. The resulting fluorescence (Fig. 7(b)) would arise almost exclusively from the etheno-dAMP, and an optical filter which passes 385-485
35 nm would further aid in the discrimination. The sample would then be illuminated with, say, 300 nm (Fig. 7(c)), and an optical filter transmitting 330 nm - 385 nm switched into the

path of the detected fluorescence, so that the detected signal would be due almost exclusively to the dGMP-photoproduct. Additionally, a time-resolved fluorescent image could also be formed, since the fluorescence decay time 5 for etheno-dAMP (20 nsec), and for dGMP-photoproduct (6.8 nsec) are quite different, and a software decision be made that at a particular spatial location, either dAMP was detected or dGMP was detected or no nucleotide was detected.

As will be apparent to those skilled in the art, 10 numerous modifications may be made in the above described embodiments within the spirit and scope of the invention.

Table I. Spectroscopic data for fluorescent products of DNA bases.

| base | product | Quantum Yield | $\lambda_{abs}(nm)$ | $\lambda_{em}(nm)$ | $\tau_1(nsec)$ | ref. |
|------|--------------------------------|----------------|---------------------|--------------------|----------------|-------|
| 15 A | etheno-A (1) | 0.56 | 300 | 415 | 20 | 8,9 |
| C | 2-acetylami no-etheno-C (2) | 0.85 | 305 | 385 | 4 | 10 |
| 20 G | 2AP (3) | 0.95 | 303 | 370 | 10 | 11,12 |
| T | d5 (4) | 0.35 (est.) | 315 | 375 | 4 | 13-15 |
| 25 | | | | | | |

(1) 1, N⁶-ethenoadenosine

(2) 2-acetylami-3, N⁴-ethenocytidine (or 2-acetylami-ethenocytidine)

(3) 2-aminopurine (or 2AP)

(4) 5-methyl-2-pyrimidinone 1- β -D-2'-deoxyriboside (or d5)

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What is claimed is:

1. A method of identifying first and second types of nucleotides in a sequence of nucleotides cleaved one-at-a-time from an oligonucleotide comprising the steps of:
 - selectively converting the nucleotides of the first type to first products and the nucleotides of the second type to second products, said products having enhanced fluorescence, said products being affixed to a substrate, each product at a different position on the substrate;
 - illuminating the substrate with electromagnetic radiation to cause the first and second products to fluoresce; and
 - detecting the fluorescence of the first and second products as a function of the position of the first and second products on the substrate, thereby identifying the position of the first and second types of nucleotides in the sequence of cleaved nucleotides.
2. A method of identifying first and second types of nucleotides in a sequence of nucleotides cleaved one-at-a-time from an oligonucleotide comprising the steps of:
 - affixing the cleaved nucleotides to a substrate, each nucleotide being affixed to the substrate at a different position on the substrate;
 - selectively converting the nucleotides of the first type to first products having enhanced fluorescence;
 - illuminating the substrate with electromagnetic radiation to cause the first products to fluoresce;
 - detecting the fluorescence of the first products as a function of their position on the substrate;
 - selectively converting the nucleotides of the second type to second products having enhanced fluorescence;
 - illuminating the substrate with electromagnetic radiation to cause the second products to fluoresce; and
 - detecting the fluorescence of the second products as a function of their position on the substrate, thereby

identifying the position of the nucleotides of the first and second types in the sequence of cleaved nucleotides.

3. A method for identifying the guanine
5 nucleotide in a sequence of nucleotides cleaved one-at-a-time from an oligonucleotide comprising the steps of:
- affixing the cleaved nucleotides to a substrate,
each nucleotide being affixed to the substrate at a different position on the substrate;
 - 10 selectively converting the guanine nucleotides to guanine products having enhanced fluorescence;
 - illuminating the substrate with electromagnetic radiation to cause the guanine products to fluoresce; and
 - detecting the fluorescence of the guanine products
15 as a function of their position on the substrate, thereby identifying the position of the guanine nucleotides in the sequence of cleaved nucleotides.

4. A method for identifying the cytosine
20 nucleotide in a sequence of nucleotides cleaved one-at-a-time from an oligonucleotide comprising the steps of:
- affixing the cleaved nucleotides to a substrate,
each nucleotide being affixed to the substrate at a different position on the substrate;
 - 25 selectively converting the cytosine nucleotides to cytosine products having enhanced fluorescence;
 - illuminating the substrate with electromagnetic radiation to cause the cytosine products to fluoresce; and
 - detecting the fluorescence of the cytosine products
30 as a function of their position on the substrate, thereby identifying the position of the cytosine nucleotides in the sequence of cleaved nucleotides.

5. A method for identifying the adenine
35 nucleotide in a sequence of nucleotides cleaved one-at-a-time from an oligonucleotide comprising the steps of:

affixing the cleaved nucleotides to a substrate,
each nucleotide being affixed to the substrate at a different
position on the substrate;

selectively converting the adenine nucleotides to
5 adenine products having enhanced fluorescence;

illuminating the substrate with electromagnetic
radiation to cause the adenine products to fluoresce; and

detecting the fluorescence of the adenine products
as a function of their position on the substrate, thereby
10 identifying the position of the adenine nucleotides in the
sequence of cleaved nucleotides.

6. A method for identifying the thymine
nucleotide in a sequence of nucleotides cleaved one-at-a-time
15 from an oligonucleotide comprising the steps of:

affixing the cleaved nucleotides to a substrate,
each nucleotide being affixed to the substrate at a different
position on the substrate;

selectively converting the thymine nucleotides to
20 thymine products having enhanced fluorescence;

illuminating the substrate with electromagnetic
radiation to cause the thymine products to fluoresce; and

detecting the fluorescence of the thymine products
as a function of their position on the substrate, thereby
25 identifying the position of the thymine nucleotides in the
sequence of cleaved nucleotides.

7. A method for base-at-a-time sequencing of an
oligonucleotide comprising the steps of:

30 (a) cleaving nucleotides one-at-a-time from the
oligonucleotide;

(b) affixing the cleaved nucleotides to a
substrate, each nucleotide being affixed to the substrate at
a different position on the substrate;

35 (c) selectively converting nucleotides of a first
type to first products having an enhanced fluorescence;

- (d) illuminating the substrate with electromagnetic radiation to cause the first products to fluoresce;
- (e) detecting the fluorescence of the first
5 products as a function of the position of the first products on the substrate;
- (f) selectively converting nucleotides of a second type to second products having an enhanced fluorescence;
- (g) illuminating the substrate with
10 electromagnetic radiation to cause the second products to fluoresce;
- (h) detecting the fluorescence of the second products as a function of the position of the second products on the substrate;
- (i) selectively converting nucleotides of a third
15 type to third products having an enhanced fluorescence;
- (j) illuminating the substrate with electromagnetic radiation to cause the third products to fluoresce;
- (k) detecting the fluorescence of the third
20 products as a function of the position of the third products on the substrate;
- (l) selectively converting nucleotides of a fourth type to fourth products having an enhanced fluorescence;
- (m) illuminating the substrate with
25 electromagnetic radiation to cause the fourth products to fluoresce;
- (n) detecting the fluorescence of the fourth products as a function of the position of the fourth products
30 on the substrate.

8. A method for base-at-a-time sequencing of an oligonucleotide comprising the steps of:

- cleaving nucleotides one-at-a-time from the
35 oligonucleotide;
- selectively converting at least the nucleotides of a first type to first products and the nucleotides of a

second type to second products, said products having enhanced fluorescence, said products being affixed to a substrate, each product at a different position on the substrate, in the order in which the nucleotides were cleaved from the
5 oligonucleotide;

illuminating the substrate with electromagnetic radiation to cause the first and second products to fluoresce; and

detecting the fluorescence of the first and second
10 products as a function of the position of the first and second products on the substrate, thereby identifying the position of the first and second types of nucleotides in the sequence of cleaved nucleotides.

15 9. A method for base-at-a-time sequencing of an oligonucleotide comprising the steps of:

cleaving nucleotides one-at-a-time from the oligonucleotide;

selectively converting at least the nucleotides of
20 a first type to first products, the nucleotides of a second type to second products, and the nucleotides of a third type to third products, said products having enhanced fluorescence, said products being affixed to a substrate, each product at a different position on the substrate, in the
25 order in which the nucleotides were cleaved from the oligonucleotide;

illuminating the substrate with electromagnetic radiation to cause the first, second and third products to fluoresce; and

30 detecting the fluorescence of the first, second and third products as a function of the position of the first, second and third products on the substrate, thereby identifying the position of the first, second and third types of nucleotides in the sequence of cleaved nucleotides.
35

10. A method for base-at-a-time sequencing of an oligonucleotide comprising the steps of:

cleaving nucleotides one-at-a-time from the oligonucleotide;

selectively converting the nucleotides to products having enhanced fluorescence, each type of nucleotide being
5 converted to a product having a distinctive fluorescence, said products being affixed to a substrate, each product at a different position on the substrate, in the order in which the nucleotides were cleaved from the oligonucleotide;

illuminating the substrate with the electromagnetic
10 radiation to cause the products to fluoresce; and

detecting the fluorescence of the products as a function of the position of the products on the substrate, thereby identifying the position of the nucleotides in the sequence of cleaved nucleotides.

15

11. In combination:

a substrate having a metal oxide surface; and
affixed to said metal oxide surface a sequence of conversion products having different fluorescent
20 characteristics, said conversion products being formed by the steps of:

cleaving nucleotides one-at-a-time from an oligonucleotide;

affixing the nucleotides to the metal oxide
25 surface in the order in which they are cleaved; and

selectively converting different types of nucleotides to said conversion products having different fluorescent characteristics.

30 12. The combination of claim 11 wherein the metal oxide surface is alumina.

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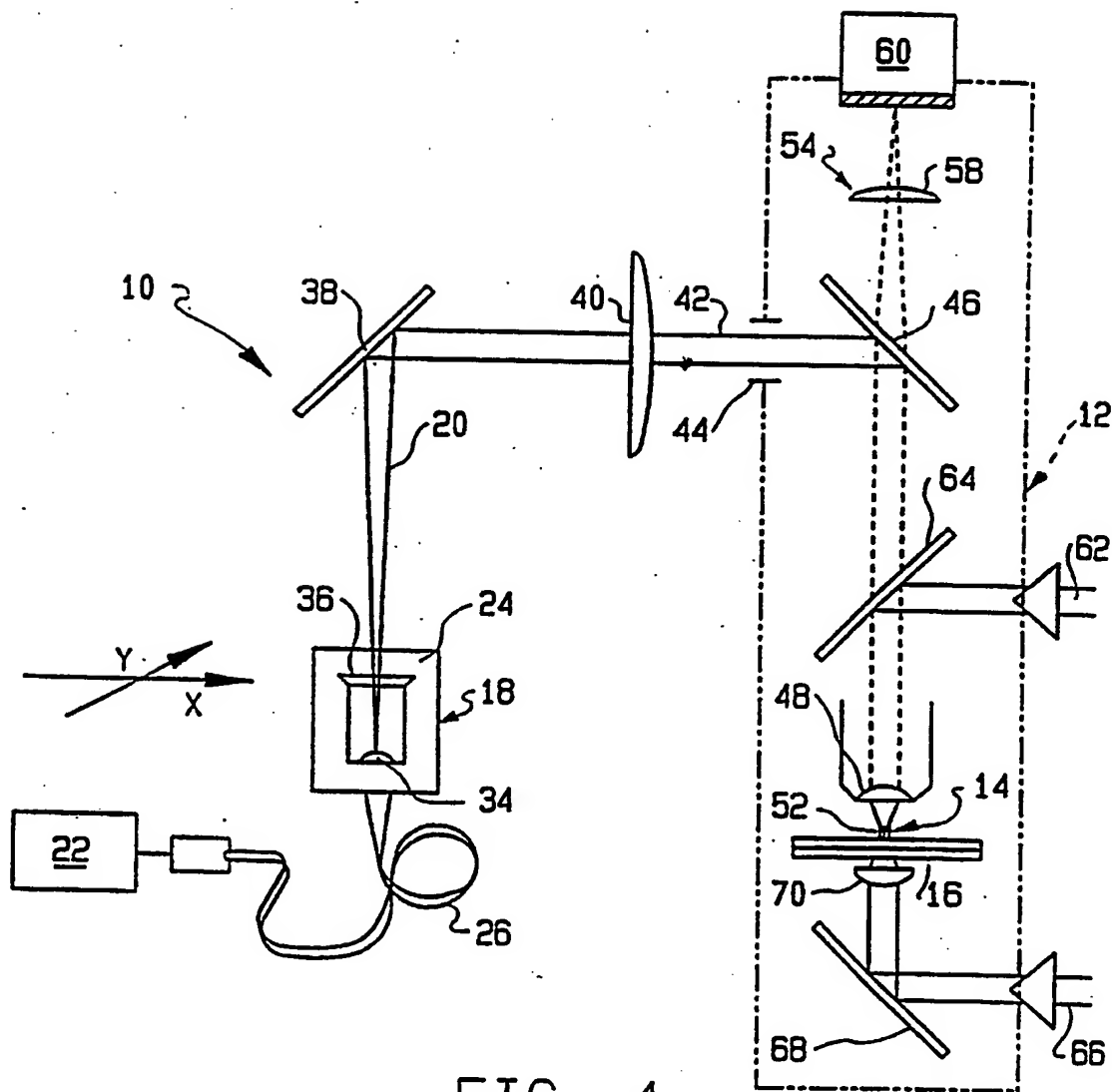


FIG. 1

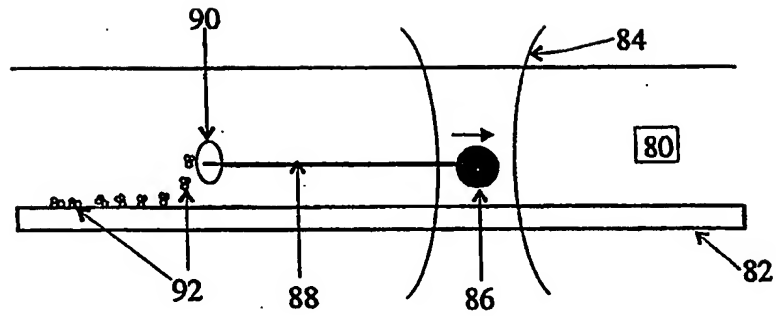


FIG. 2(a)

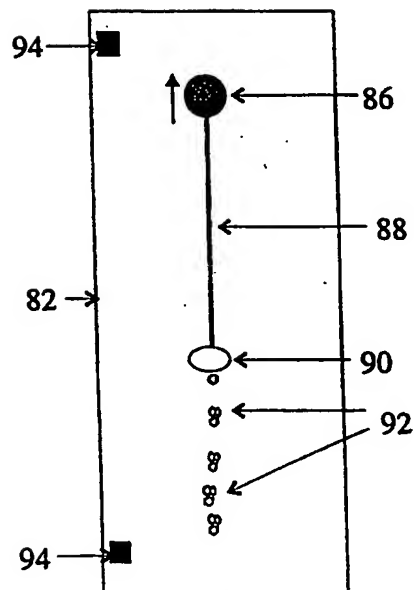


FIG. 2(b)

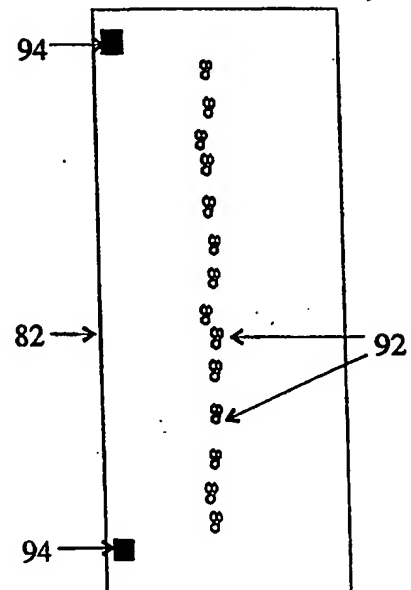


FIG. 2(c)

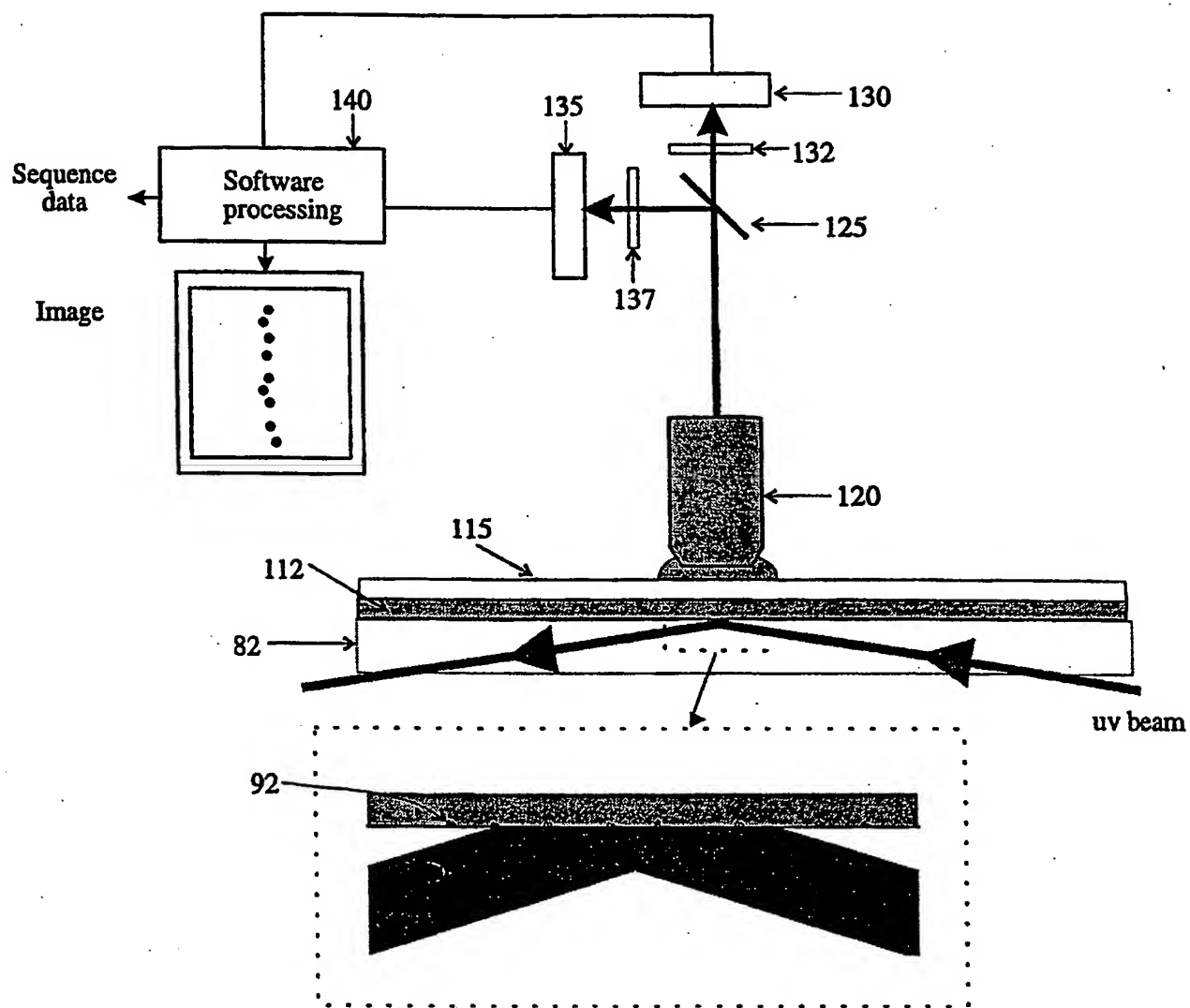


FIG. 3

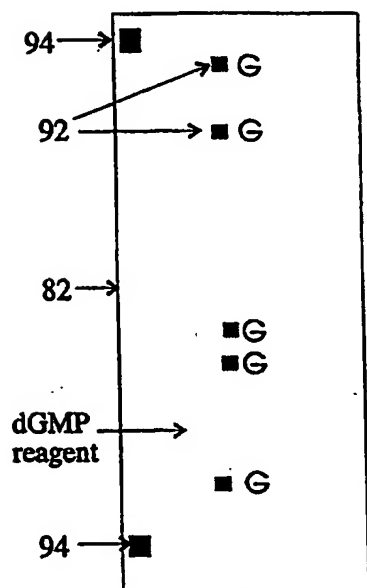


FIG. 4(a)

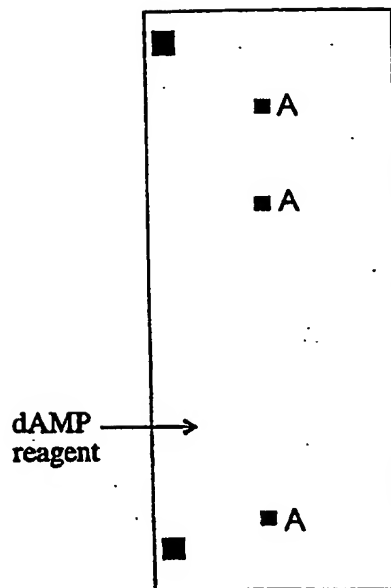


FIG. 4(b)

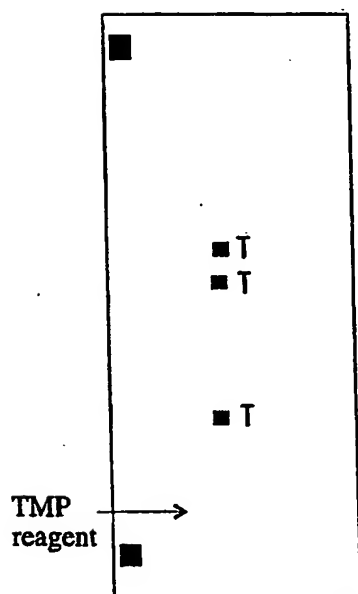


FIG. 4(c)

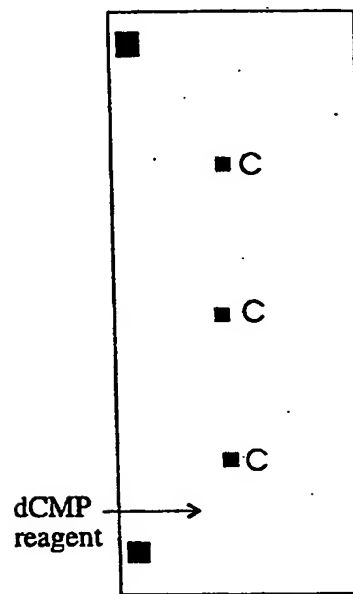


FIG. 4(d)

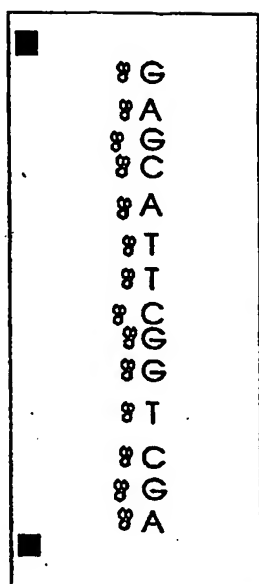


FIG. 4(e)

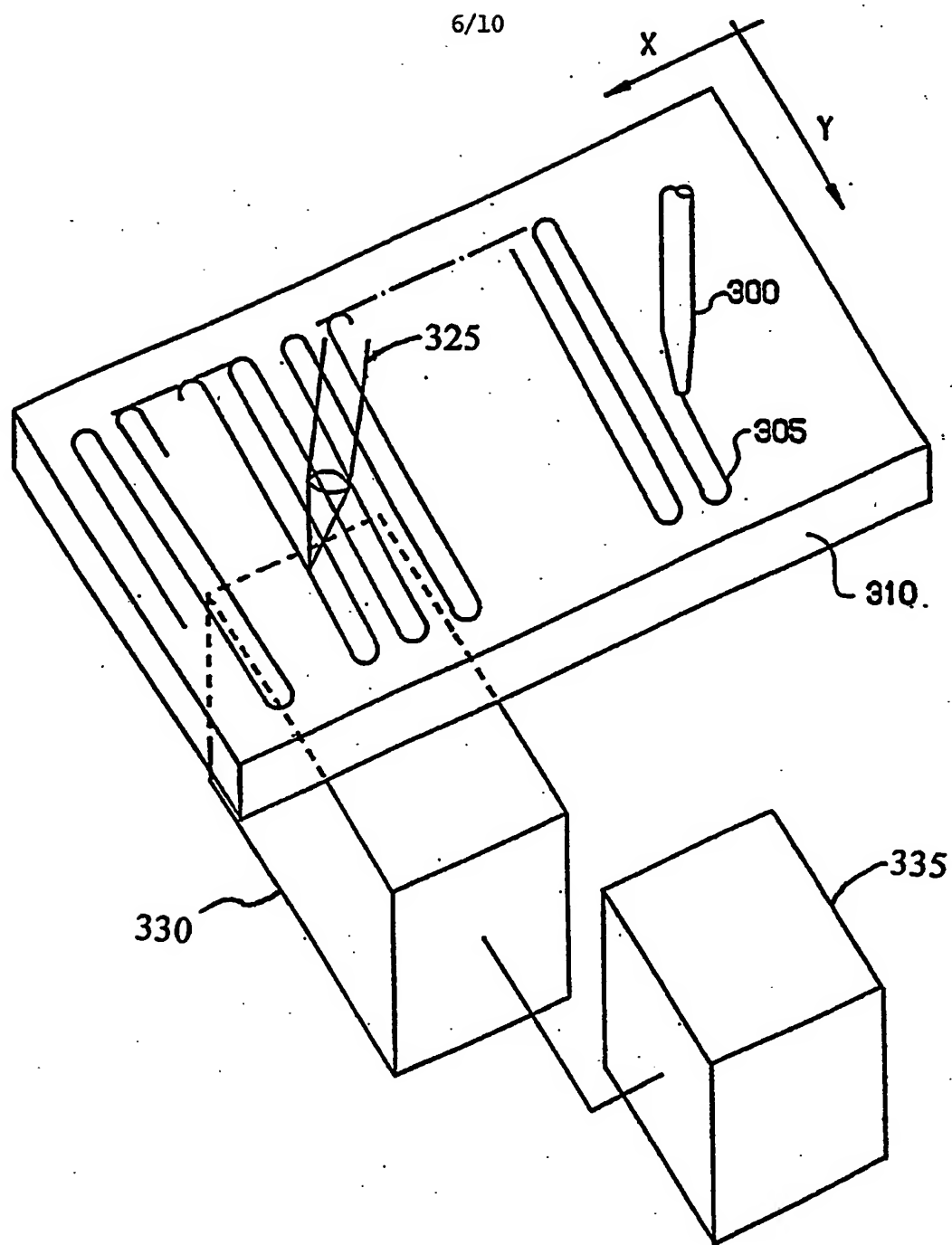


FIG. 5

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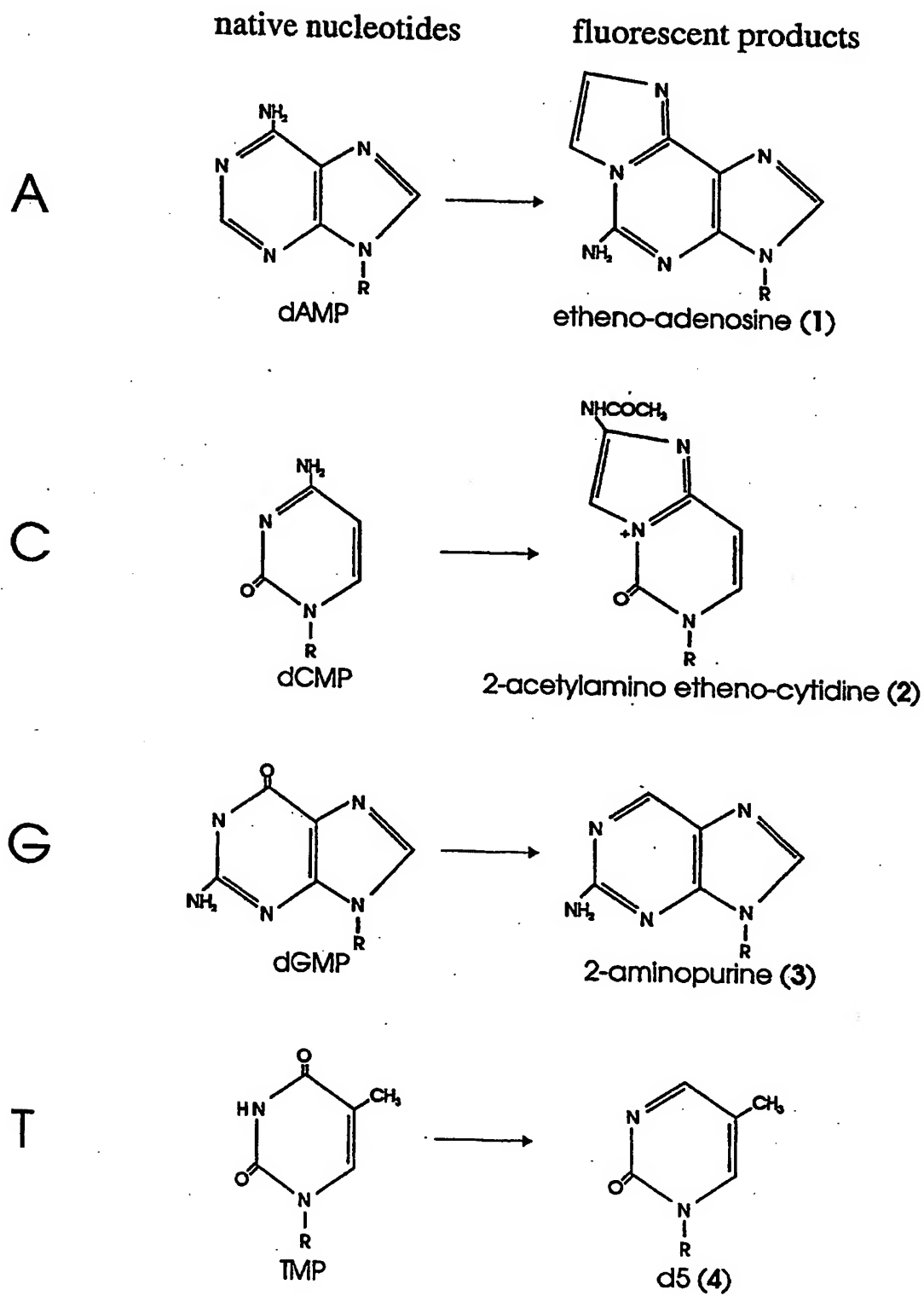


Fig. 6

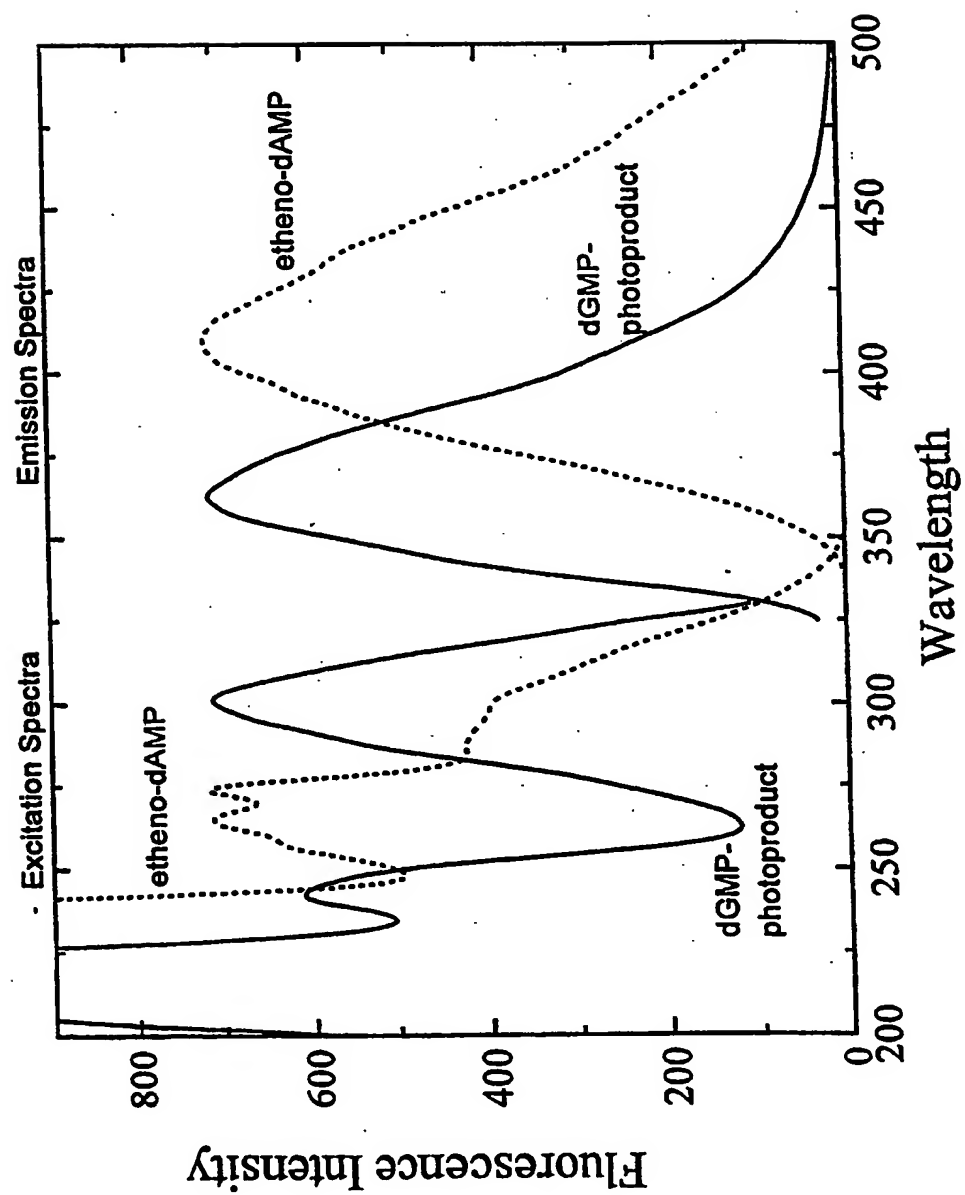


FIG. 7(a)

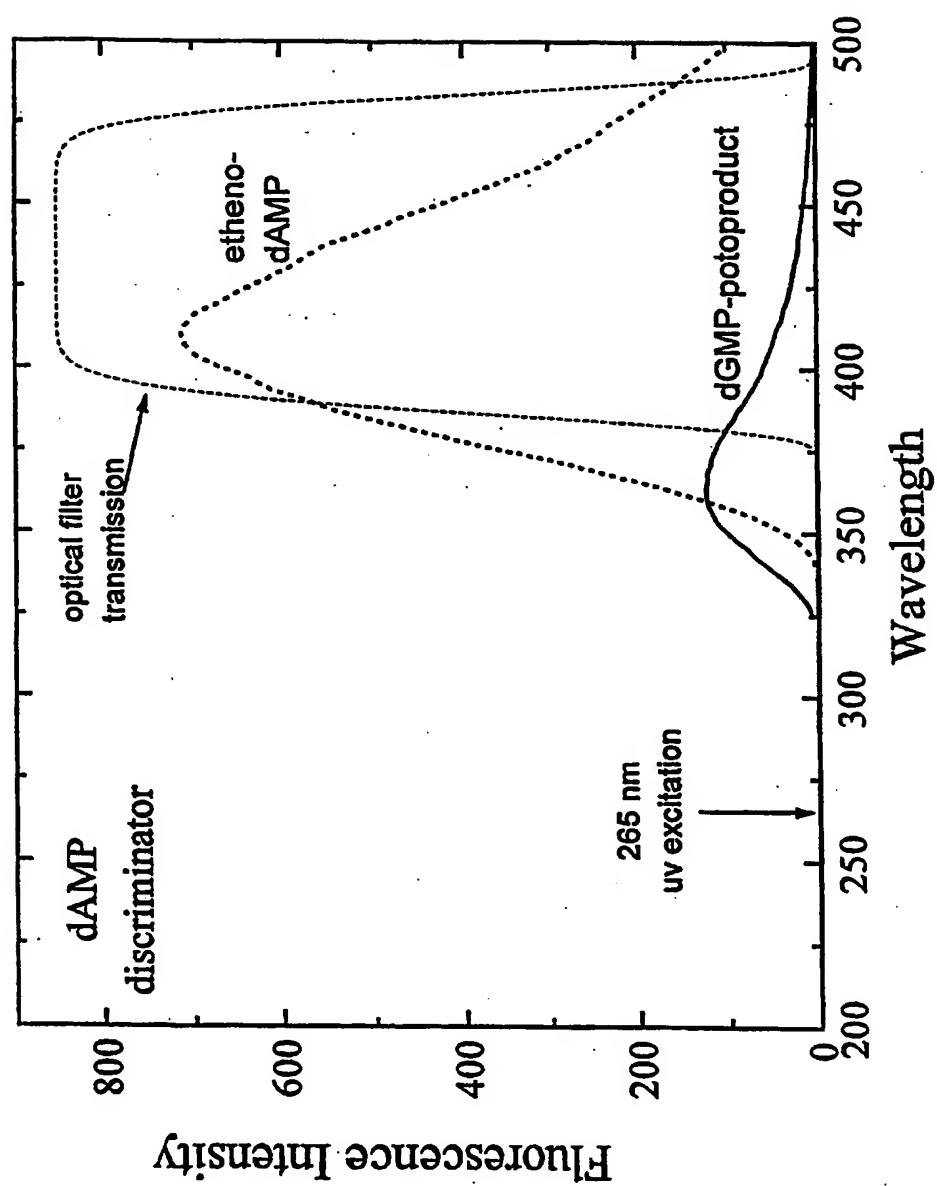


FIG. 7(b)

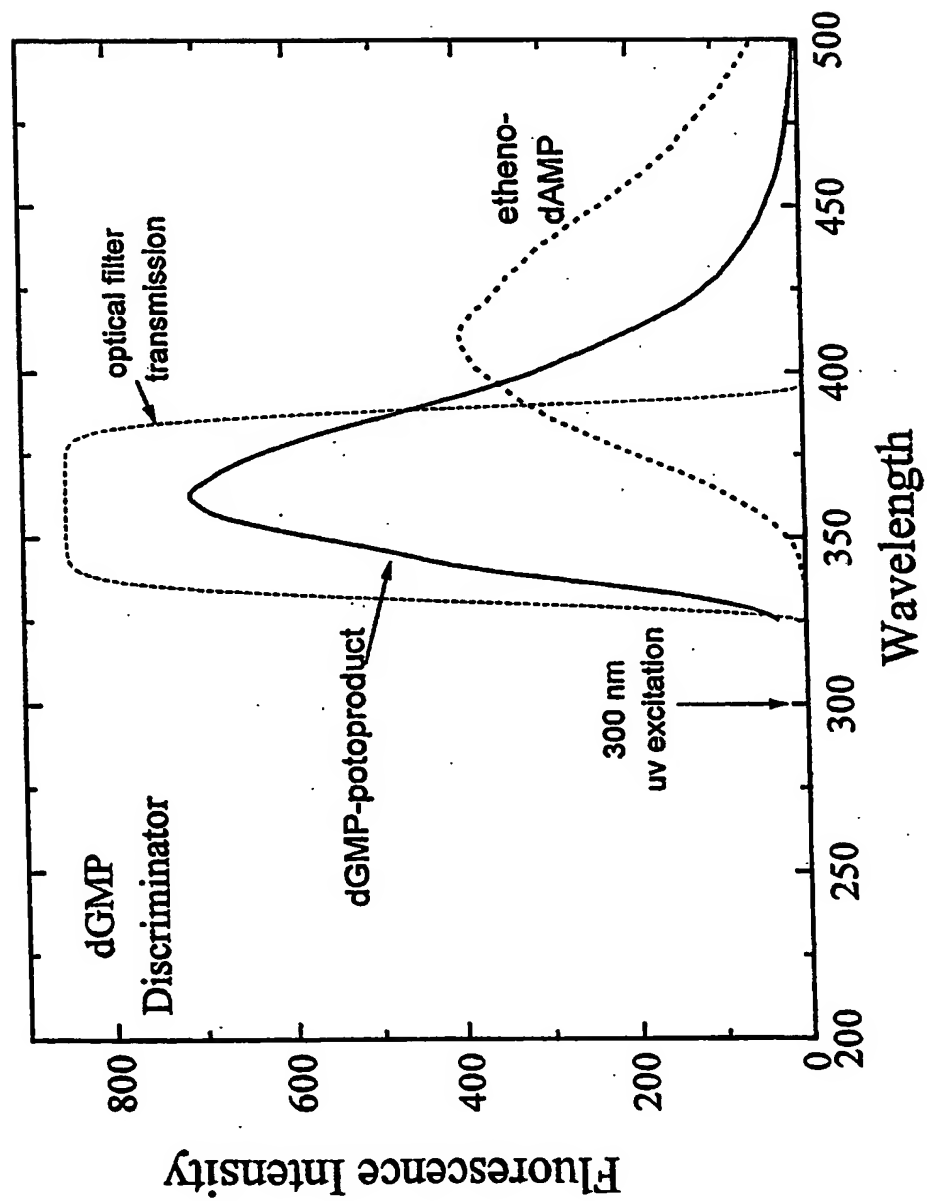


FIG. 7(c)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18808

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/00, 21/04

US CL :435/6; 536/24.3, 26.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/24.3, 26.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, HCAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| Y | US 5,525,464 A (DRMANAC et al.) 11 June 1996, see entire document. | 1-12 |
| Y | WO 95/21266 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 10 August 1995, see entire document. | 1-12 |

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Date of the actual completion of the international search
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